

A Femtosecond Laser Creates a Stronger Flap than a Mechanical Microkeratome

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PURPOSE. To compare corneal flaps made with a femtosecond (FS) laser with those made with a mechanical microkeratome (MM) in rabbits, measuring early postoperative inflammation and apoptosis and late postoperative adhesion strength.

METHODS. Study 1 involved four treatment groups: one with flaps made only with the FS laser (FS group), one with flaps made with the FS laser followed by excimer laser ablation (FS+LASIK), one with flaps made with the MM, and one with flaps made with the MM followed by excimer laser ablation (MM+LASIK). The eyes were analyzed by histology and TUNEL staining for apoptosis at 4 and 24 hours. Study 2 involved two reference groups: an FS group and an MM group. Adhesion strength was measured with a tension meter 1 and 3 months later.

RESULTS. Study 1: Inflammatory cell infiltration in the central cornea was significantly greater in the FS group than in the MM group at 4 and 24 hours ($P < 0.05$) and was significantly greater in the FS+LASIK group than in the MM+LASIK group at 24 hours ($P < 0.05$). Infiltration at the peripheral interface was significantly greater in the FS group than in the MM group and was significantly greater in the FS+LASIK group than in the MM+LASIK group at 24 hours ($P < 0.05$). Study 2: At 1 and 3 months, 126.7 and 191.3 grams of force (gf) were needed to detach the flaps in the FS group, compared with 65 and 127.5 gf in the MM group, respectively. The grams of force needed was significantly higher in the FS group than in the MM group at 3 months ($P < 0.05$).

CONCLUSIONS. The FS laser produces greater corneal stromal inflammation than the MM early postoperatively without any increase in apoptosis and stronger flap adhesion late postoperatively. Therefore, it may require stronger anti-inflammatory drugs to be administered. (*Invest Ophthalmol Vis Sci*. 2006;47:599–604) DOI:10.1167/iovs.05-0458

The corneal wound-healing response after refractive surgery is extremely complex. Apoptotic keratocytes and inflammatory cell infiltrations are the earliest changes noted in the cornea when there is injury to the epithelium.^{1–3} Apoptosis is delayed and detected to be at its maximum at 4 hours after

injury, when assayed with terminal deoxyribonucleotide transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL).⁴ The remaining keratocytes surrounding the apoptotic zone and begin to undergo proliferation within 12 to 24 hours (Hutchison SR et al. *IOVS* 1999;40:ARVO E-Abstract 622). These proliferating cells give rise to wound-healing keratocyte-derived cells called myofibroblasts.^{5,6} Cytokines produced by myofibroblasts, such as fibroblast growth factor (FGF), keratocyte growth factor (KGF), and transforming growth factor (TGF)- β , regulate the proliferation, migration, and differentiation of the overlying healing epithelium.^{7,8} Polymorphonuclear leukocytes (PMNs) are the first cells to migrate into the corneal tissues in response to injury. They are highly differentiated cells that are unable to proliferate⁹ and are found in the limbus and wounded areas within the first 2 days after corneal injuries such as superficial epithelial defects and alkaline burns.^{10,11} The density of PMNs starts to decline by the third day, and other cell types, such as mononuclear phagocytes appear along with signs of new capillaries.¹²

Apoptosis in keratocytes and inflammatory cell infiltrations occur immediately beneath the healing epithelium after photorefractive keratectomy (PRK). These phenomena occur at the level of the interface, further from the overlying epithelium, after laser-assisted in situ keratomileusis (LASIK). Epithelial hyperplasia appears to be lower after LASIK than PRK, even though epithelial hyperplasia can occur after LASIK.¹³ As epithelium-derived cytokines appear to be the inducers of keratocyte apoptosis, the surgeon should seek to minimize the introduction of epithelial tissue into the interface. Even though LASIK is best performed with sharp blades that incise the epithelium with minimal trauma, a femtosecond (FS) laser (e.g., the IntraLase FS; IntraLase, Irvine, CA) laser could be more useful in producing a flap with uniform thickness and minimal introduction of epithelial debris and cytokines into the interface.¹⁴

In vitro studies using cultures of human corneal fibroblasts have shown that TGF β and epidermal growth factor (EGF) both stimulate the chemotaxis of corneal fibroblasts, and EGF enhances DNA synthesis.^{15,16} They also increase the synthesis of collagen and fibronectin. Treatments with these growth factors probably increase the tensile strength of corneal incisions by influencing key processes of scar formation: increasing the chemotactic migration of corneal fibroblasts to the site of a stromal injury, increasing mitosis of fibroblasts, and stimulating synthesis of collagen. During corneal wound healing after LASIK, various cytokines, including TGF β and epidermal growth factor (EGF), are released into the interface. These factors may affect the adhesive strength of flaps made by different keratomes. Therefore, it has been postulated that flaps created with FS lasers may show less inflammatory cell infiltration and apoptosis because of improved epithelial preservation at the early postoperative period and may have weaker adhesion strength during the late postoperative period than those created with a mechanical microkeratome (MM).¹⁴

The goals of this study were to compare the effects of an FS laser and an MM on corneal wound healing in an animal model. We determined inflammatory cell infiltration and apoptosis

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around corneal flaps at the early postoperative period and the adhesion strength of flaps at the late postoperative period. To our knowledge, this is the first study using an animal model to compare these methods.

METHODS

Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS), trypsin-EDTA, and penicillin-streptomycin were purchased from Invitrogen-Life Technologies (Gaithersburg, MD); the TUNEL staining kit from Roche Molecular Biochemicals (Mannheim, Germany); and all chemicals (biotechnology grade) from Amresco (Solon, OH).

Study 1: Procedures

The first study involved four reference groups: one (FS group, $n = 12$) with flaps made with an FS laser (IntraLase Corp.); one (FS+LASIK group, $n = 12$) with flaps made with an FS laser followed by excimer laser ablation; one (MM group, $n = 12$) with flaps made with an automated corneal shaper (ACS; Bausch & Lomb, Rochester, NY); one (MM+LASIK group, $n = 12$) with flaps made with an MM followed by excimer laser ablation; and a control group ($n = 12$) in which no procedure was performed. Untreated left eyes of rabbits randomly assigned four to a group were used as the control.

Animals and Preparation. Procedures were conducted in conformity with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. New Zealand albino rabbits weighing 1.5 to 2 kg were used. Each rabbit was anesthetized intramuscularly with xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (50 mg/kg) and received 0.5% proparacaine hydrochloride topical eye drops (Alcon-Couvreur, Puurs, Belgium) with 0.1% levofloxacin (Santen Pharmaceutical Co., Osaka, Japan). Each right eye was positioned anterior to the eyelids and temporarily retained in that conformation by clamping the temporal upper and lower eyelids together with a mosquito clamp.

FS Laser Procedure. An FS laser was used to make a flap in the FS and FS+LASIK groups. We attempted to hold the anesthetized rabbit's head still and a flat, glass contact lens was used to appenate the cornea without using a suction ring. The initial settings were a planned flap diameter of 8.0 mm, a flap thickness of 110 μm , a hinge angle of 50°, a flap energy of 3.2 μJ , a beam separation of 10 \times 11 μm , and a side-cut energy of 3.2 μJ . After the cornea had been appenated with the disposable glass lens, a contact lens cone attached to the FS laser was lowered into position over the eye. When the settings were acceptable, the laser was activated, and the ablation began from the hinge in a raster pattern, moving horizontally. The hinge location was superior in all cases. After waiting 5 minutes to allow the absorption of microcavitation bubbles, we used a spatula to separate the flap by breaking microadhesions in the laser wound in the temporal quadrant near the hinge. The spatula was wiped free of epithelium, placed in the initial location, advanced 3.0 mm, and swept inferiorly. The interface was opened in one to three such passes, and the flap was then reflected superiorly. One drop of 0.1% levofloxacin was placed in the interface, and the flap was repositioned using the spatula. Three 10-0 nylon sutures were used to inhibit detachment of the flap at the nasal, temporal, and inferior interfaces. After the mosquito clamp was removed from the eyelid, tarsorrhaphy was performed in each treated eye and continued for the first 4 and 24 hours.

Mechanical Microkeratotomy. In the MM and MM+LASIK groups, a microkeratome (ACS; Bausch & Lomb) was used to make a flap. A corneal flap (130 μm thick) was made with a nasal hinge position in the right eye of all rabbits, by using the ACS microkeratome without a suction ring. A new blade was used for each rabbit. A spatula was used to free the laser wound in the temporal quadrant near the

hinge. One drop of 0.1% levofloxacin was placed in the interface, and the flap was repositioned with the spatula. The remaining procedures were as described for the laser method.

Excimer Laser Ablation. In the FS+LASIK and MM+LASIK groups, after a flap was created, excimer laser ablation was performed (Zyoptix 217z laser; Bausch & Lomb) at 10 Hz with a 5.0-mm optical zone. The intended correction was -10 D. After ablation, the interface was cleaned with a dry cellulose sponge. One drop of 0.1% levofloxacin was placed in the interface, and the flap was repositioned with a spatula. The remaining procedures were the same as for the FS laser ablation.

Tissue Processing and Analysis. The rabbits were euthanized by CO₂ inhalation at 4 and 24 hours after the procedures. Eyes were immediately enucleated and fixed in 4% paraformaldehyde for 24 hours. The corneas were excised, bisected, embedded in paraffin wax, and sectioned at 5 μm . Sections were subjected to hematoxylin and eosin (H&E) staining and TUNEL assay. After H&E staining, the numbers of inflammatory cells in the corneal stroma around the flap were counted in three fields of three tissue slides for each specimen under a phase-contrast microscope (Olympus, Tokyo, Japan). Counting was performed at the central cornea and peripheral interface. To eliminate any possible investigator bias, this procedure was performed in a blinded fashion on serially numbered slides.

TUNEL Assay. To evaluate the degree of apoptosis, we performed a TUNEL assay with a fluorescence apoptosis detection system, according to the manufacturer's instructions (Roche). Four sections from each specimen were evaluated with an inverted microscope (DMIRE2; Leica, Deerfield, IL) equipped with a confocal laser scanning system (TSC-SP2; Leica, Heidelberg, Germany) at high magnification ($\times 400$) to count TUNEL-positive keratocytes around the flap.

Study 2: Measurement of Adhesion Strength

The second study used two groups of rabbits: the FS group with flaps made with an FS laser ($n = 8$), and the MM group with flaps made with an MM ($n = 8$). Corneal flaps were made as just described. One drop of 0.1% levofloxacin was placed in the interface, and the flap was repositioned with a spatula. The remaining procedures were as described.

Measurement of Adhesion Strength. Rabbits were euthanized by CO₂ inhalation at 1 and 3 months after the procedures. We located the edge of the flap with a microscope and separated enough of it with a curved mosquito clamp to permit the attachment of a 6-0 black silk loop suture, also gripped by a mosquito clamp. The length of the loop suture was 10 cm. A tension meter (Attonic, Aichi, Japan) was used to measure the adhesion strength, and flap movement was observed by microscope. When the loop suture was pulled with a continuous load, the center of the flap lifted, and the edge of the flap started to slip. Finally, the entire flap became detached from the corneal bed. The load at flap detachment was measured, and the values were compared between groups.

Statistical Analyses

Data are expressed as the mean \pm SE. Statistical comparisons of inflammatory cell and number of apoptotic cells were performed with Student's *t*-tests and ANOVA. Adhesion strengths were analyzed with nonparametric Mann-Whitney tests. The level of significance was taken as $P < 0.05$.

RESULTS

Study 1: Histologic Findings

At the central cornea, the number of inflammatory cells was significantly greater than in the control in all groups at 4 and 24 hours, except in the FS+LASIK group at 4 hours and in the MM+LAISK group at 24 hours (Table 1; Figs. 1, 2, 3; $P < 0.05$).

TABLE 1. The Number of Inflammatory Cells around the Corneal Flap

Area	Time (h)	Groups			
		Control	FS	FS + LASIK	MM
Central cornea	4	0 ± 0	11.4 ± 3.0**	1.5 ± 0.9*	3.4 ± 1.5*
	24	0 ± 0	27.8 ± 2.8***	11.7 ± 2.1**	6.0 ± 1.2*
Peripheral interface	4	0 ± 0	49.4 ± 3.4	42.2 ± 2.3	39.8 ± 4.6
	24	0 ± 0	80.5 ± 5.8***	47.7 ± 3.9**	38.5 ± 5.2**

Data are expressed as mean cells per $\times 400$ field \pm standard error.

***, **, * Significant differences between those groups at a particular time ($P < 0.05$).

Infiltration at the peripheral interface was also significantly greater than controls in all groups ($P < 0.05$).

At 4 hours, inflammatory cell infiltration at the central cornea was significantly greater in the FS group than in the MM group ($P < 0.05$). It was also significantly greater in the FS group than in the FS+LASIK group at 4 hours ($P < 0.05$). At 24 hours, infiltration at the central cornea was significantly greater in the FS group than in the MM group, in the FS+LASIK group than in the MM+LASIK group and in the FS group than in the FS+LASIK group ($P < 0.05$). At 24 hours, infiltration at the peripheral interface was significantly greater in the FS group than in the MM group, in the FS+LASIK group than in the MM+LASIK group, in the FS group than in the FS+LASIK group, and in the MM group than in the MM+LASIK group ($P < 0.05$).

Evaluation of Apoptosis. There were significantly more apoptotic cells in the central cornea in every group at 4 and 24 hours than in the control (Table 2; Figs. 1, 2, 4. $P < 0.05$). There were no statistically significant differences between groups, except that there were significantly more apoptotic cells in the FS group than in the FS+LASIK group at 4 hours ($P < 0.05$).

Study 2: Adhesion Strength

In the FS group, 126.7 and 191.3 g of force (gf) were needed to separate the flap at 1 and 3 months after the procedure, respectively; in the MM group the corresponding values were 65 and 127.5 gf (Fig. 5). The adhesion strength increased marginally over time in both groups ($P = 0.09$ and 0.11 in the FS and MM groups, respectively). The adhesion strength in the

FS group was marginally higher than in the MM group at 1 month ($P = 0.1$), but was significantly higher at 3 months ($P < 0.05$).

DISCUSSION

FS lasers provide potentially safe advantages over the MM approaches, because they cut flaps with uniform thickness. These avoid the central thinning and entrance hinge disparity associated with blade cutting.¹⁴ Moreover, flap size and thickness, edge angle, hinge width, and locations are easily varied.¹⁴ The flap diameter and hinge size and location are more predictable than those with microkeratomes, for which the standard deviation of the flap diameter is 0.3 mm.¹⁷ Suction rings and applanation glasses are disposable for the FS laser, possibly decreasing contamination and saving time used for sterilization. Above all, improved epithelial preservation is a significant safety advantage for the FS laser.¹⁴

The disadvantages of an FS laser are that the surface of stromal bed created with the FS laser was not as smooth as that produced with an MM.¹⁸ Also, it was recently demonstrated by scanning electron microscopy (SEM) that surface irregularity was greater in the bed of lamellar keratotomies created with FS lasers than in those created with MMs.¹⁹ Two slipped flaps and 20 cases of interface inflammation were reported in a series that included 103 eyes.²⁰ One case of macular hemorrhage after LASIK with an FS laser flap creation has been reported.²¹

It has been postulated that corneal flaps created by FS lasers could produce less corneal stromal inflammation and apoptosis in the early postoperative period because of improved epithelial preservation and that they might have weaker adhesion

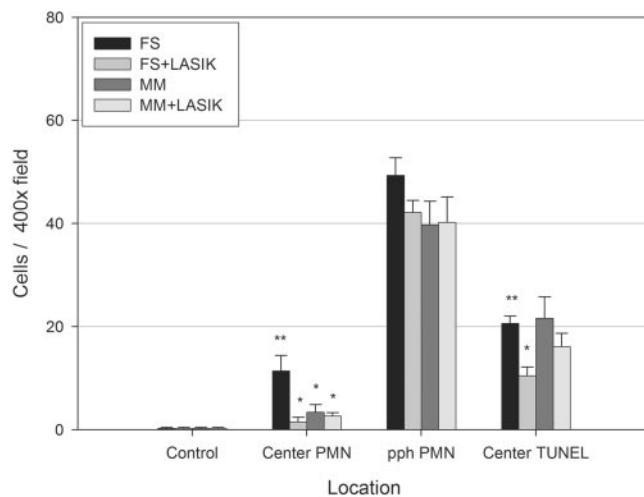


FIGURE 1. Quantitation of inflammatory cells in the central cornea and peripheral interface and TUNEL-positive cells in the central cornea at 4 hours. Error bars, SEM. **, *, * Significant differences between those groups at a particular time ($P < 0.05$).

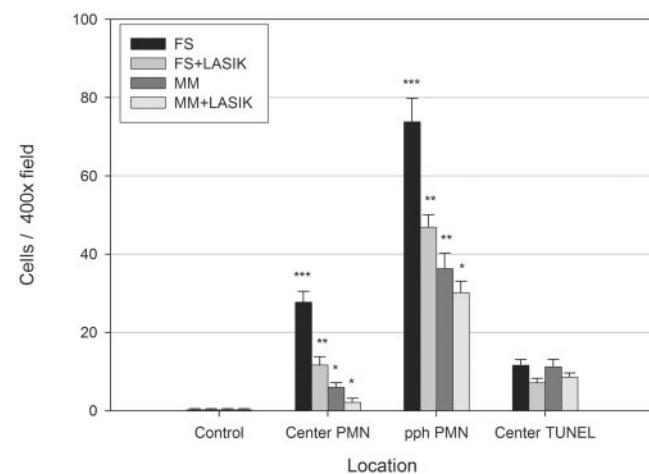


FIGURE 2. Quantitation of inflammatory cells in central cornea and peripheral interface and TUNEL-positive cells in the central cornea at 24 hours. Error bars, SEM. **, ***, *, * Significant differences between those groups at a particular time ($P < 0.05$).

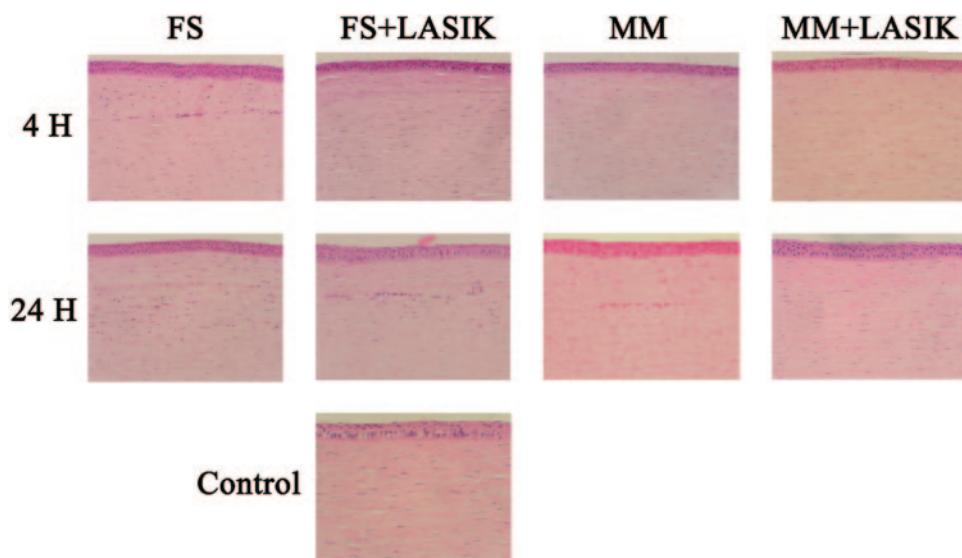


FIGURE 3. The inflammatory cell infiltration of central cornea observed by phase contrast microscopy. At 4 and 24 hours, infiltration was significantly greater in the FS group than in the MM group ($P < 0.05$). At 24 hours, infiltration was significantly greater in the FS+LASIK group than in the MM+LASIK group ($P < 0.05$, cells/ $\times 400$ field).

strength than those created with MMs in the late postoperative period.¹⁴ Our results were completely inconsistent with both of these ideas. In the early postoperative period, there was more corneal stromal inflammation in the FS and FS+LASIK groups. Such inflammatory cell infiltration into injured corneal tissue is a hallmark of wound repair.^{22,23} PMNs are the first cells to migrate into the tissue in response to injury and may enter the wound, phagocytose bacteria, secrete proteases that remove damaged extracellular matrix components, and release cytokines and growth factors. The macrophages produce β FGF, TGF α , platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF).^{10,11} PMNs also produce TGF β 1, which stimulates the proliferation of keratocytes and other factors that in turn attract macrophages to the area.⁹ Depletion of leukocytes markedly delays fibroblast proliferation,²⁴ suggesting the importance of these factors. Therefore, leukocytes are potent sources of growth factors and cytokines, all of which are necessary for the initiation and propagation of new tissue formation in wounds.²⁵

Excessive inflammatory cell infiltration or diffuse lamellar keratitis (DLK) is probably attributable to endogenous factors that trigger inflammation, mainly secondary to epithelial injury after LASIK.²⁶ Although the etiology of DLK is unknown, sev-

eral reports have linked epithelial defects, trauma, Meibomian gland secretions, and bacterial toxins with DLK.²⁷ The genesis of DLK can be associated with the release of proinflammatory cytokines from the corneal epithelium and flap interface. A platelet-activating-factor receptor antagonist was reported to block both the induction of DLK and keratocyte apoptosis.²⁸ The severity of gutter in the corneal flap margin was also associated with the incidence of DLK.²⁹ As mentioned earlier, Solomon et al.¹⁹ used SEM to show that surface irregularity was greater in the beds of lamellar keratectomies created with an FS laser than those created with an MM. Because the flap edge created with an FS laser can be deeply cut or irregular, re-epithelialization may take more time, and more inflammatory cells may infiltrate in the flap interface than with an MM. In contrast, Binder²⁰ described 20 cases of interface inflammation (DLK) in which there was no effect on visual outcome after LASIK with an FS laser.²⁰ Decreases in the side-cut energy and side-cut angle eliminated the interface inflammation. In that series, a side-cut energy of 6 to 8 μ J, which was considerably higher than the 3.2 μ J used in our study, was applied. Although it was difficult to compare the data from both studies directly, because different experimental subjects were involved, the side-cut energy could be related to inflammatory cell infiltration.

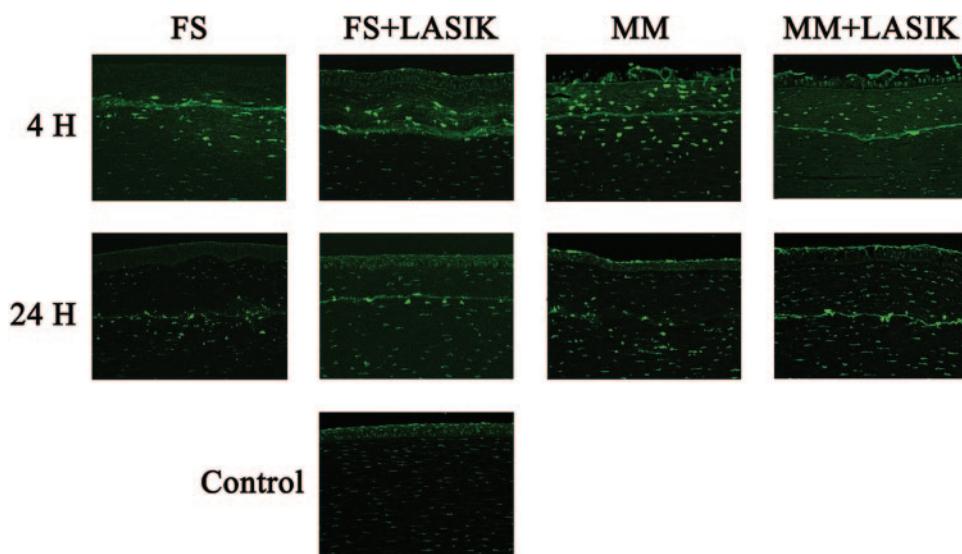


FIGURE 4. Detection of apoptosis by TUNEL assay in the central cornea of each group at 4 and 24 hours. The TUNEL-positive cells were scattered in the areas near the lamellar interface created by the keratotomy. There were no statistically significant differences between groups, except that there were significantly more apoptotic cells in the FS group than in the FS+LASIK group at 4 hours ($P < 0.05$, cells/ $\times 400$ field).

TABLE 2. The Number of Apoptotic Cells in the Central Cornea

Time (h)	Control	FS	FS + LASIK	MM	MM + LASIK
4	0.3 ± 0.3	20.6 ± 1.3**	10.5 ± 1.7*	21.7 ± 4.1	16.1 ± 2.5
24	0.3 ± 0.3	11.6 ± 1.5	7.1 ± 1.2	11.3 ± 1.9	8.6 ± 1.1

Data are expressed as mean cells per $\times 400$ field \pm standard error.

**, * Significant differences between those groups at a particular time ($P < 0.05$).

tion in the FS group. Furthermore, the higher energy of an FS laser than that of an MM could explain the more inflammatory cell infiltration in the corneal stoma in the FS group in this study. Using a lower side-cut energy can alleviate such inflammatory cell infiltration.²⁰ In particular, we found prominent inflammatory cell infiltration after FS laser LASIK using this animal model. Thus, after a flap is created with an FS laser, stronger anti-inflammatory drugs may be necessary to control postoperative inflammation.

It was also significant that the flaps made with an FS laser showed higher adhesion strengths than those created with an MM: the first such report to our knowledge. In this study, flap creation with an FS laser caused more inflammatory cell infiltration of the corneal stroma at early postoperative period. This is likely to increase flap adhesion strength later, because leukocytes are a source of growth factors and cytokines, all of which are necessary for initiation and propagation of new tissue formation in wounds.²⁵ Further studies are needed to prove such a relationship. Furthermore, when surgeons perform enhancement after LASIK, the required flap can be produced by either recutting or lifting the previous flap. Flap lifting is very easy, even several months after the first LASIK. Every surgeon experiences some cases of accidental self-removal of a flap or flap dislocation caused by trauma, even long after LASIK. Therefore, the widespread use of FS lasers could make stronger flaps that are more resistant to trauma and reduce the incidence of such a disastrous complication, but such flaps cannot be lifted easily if later corneal enhancement is needed.

It was noteworthy that inflammatory cell infiltration and apoptosis of the corneal stroma were more severe in the groups where only a flap was created than in the groups where laser ablation was performed after the creation of a flap with both FS laser and MM. This finding contrasts with that of a

previous study.³⁰ In that study, hinged corneal flaps created with an ACS MM (Bausch & Lomb) and repositioned without any laser treatment had a lower rate of apoptosis than in the LASIK group where a hinged corneal flap was created and treated by laser ablation (-5 D). We suspect this discrepancy may be attributable to the different experimental settings: the higher-energy laser ablation used in this study with the goal of correcting high myopia (-10 D) probably removed more epithelial debris, cytokines from injured epithelium, and their receptors of keratocytes posterior to the interface than did the low-energy laser ablation used in previous study. In addition, the TUNEL-positive cells were scattered in the areas anterior and posterior to the lamellar interface in the FS and MM groups. Conversely, in the FS+LASIK and MM+LASIK groups, these cells were scattered in the areas only anterior to the lamellar interface, which meant that most cytokine receptors in keratocytes posterior to the interface were removed by laser ablation (Fig. 4). Further experiments are necessary to confirm this idea.

The present study was subject to several limitations. First, the small number in each group limits interpretation of the results. Second, we were unable to determine the mechanism of stronger flap adhesion after FS laser flap creation. Therefore, further studies using larger groups, immunohistochemical staining, and longer follow-ups are necessary to clarify the relationship between strong flap adhesion and FS laser use. Third, in this study, postoperative steroid therapy was not used. Prednisolone acetate (1%)-treated cornea had less wound-healing effect in a feline cataract incision than untreated or nonsteroidal anti-inflammatory drug (NSAID)-treated cornea, because this cornea wound had less fibroblastic response.³¹ However, further study is needed to evaluate whether topical prednisolone acetate 1% can change the adhesion strength of corneal flap in our rabbit model. Fourth, there were high standard errors in the number of TUNEL-positive cells around the flap in the FS laser and MM groups. These errors could be due to artifacts, in that in addition to keratocytes, other cells present could undergo apoptosis, and in some cases necrotic cells would be labeled with the TUNEL assay.³²

In conclusion, in this rabbit model, an FS laser produced greater corneal stromal inflammation early after surgery than an MM, without any later postoperative increase in apoptosis, and flap adhesion was stronger. Therefore, the use of the FS laser method may necessitate the postoperative use of stronger NSAIDs.

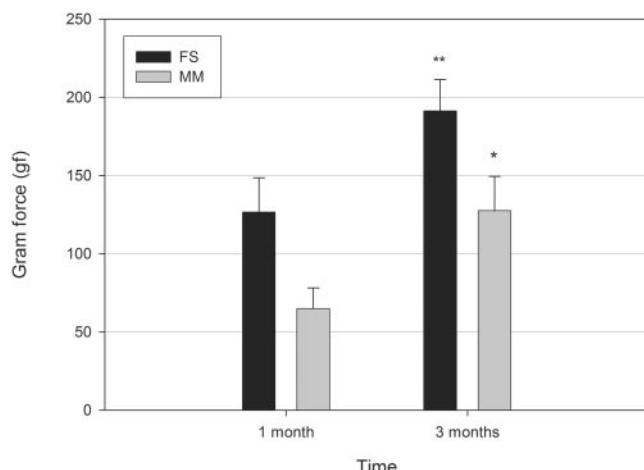


FIGURE 5. Measurements of adhesion strength. Adhesion in the FS group was marginally higher than in the MM group at 1 month ($P = 0.1$), but was significantly higher at 3 months ($P < 0.05$). **, * Significant differences between those groups at a particular time.

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